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OXIDATIVE STRESS AND THE EVOLUTION OF SEX DIFFERENCES IN LIFE SPAN AND AGEING IN THE DECORATED CRICKET, *GRYLLODES SIGILLATUS*

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The Free Radical Theory of Ageing (FRTA) predicts that oxidative stress, induced when levels of reactive oxygen species exceed the capacity of antioxidant defenses, causes ageing. Recently, it has also been argued that oxidative damage may mediate important life-history trade-offs. Here, we use inbred lines of the decorated cricket, *Gryllodes sigillatus*, to estimate the genetic (co)variance between age-dependent reproductive effort, life span, ageing, oxidative damage, and total antioxidant capacity within and between the sexes. The FRTA predicts that oxidative damage should accumulate with age and negatively correlate with life span. We find that protein oxidation is greater in the shorter lived sex (females) and negatively genetically correlated with life span in both sexes. However, oxidative damage did not accumulate with age in either sex. Previously we have shown antagonistic pleiotropy between the genes for early-life reproductive effort and ageing rate in both sexes, although this was stronger in females. In females, we find that elevated fecundity early in life is associated with greater protein oxidation later in life, which is in turn positively correlated with the rate of ageing. Our results provide mixed support for the FRTA but suggest that oxidative stress may mediate sex-specific life-history strategies in *G. sigillatus*.

KEY WORDS: **Age-dependent reproductive effort, ageing, life span, oxidative damage, protein oxidation, reactive oxygen species, sexual selection, total antioxidant capacity.**

The concept of constraint driven by resource limitation forms the cornerstone of life-history theory (Roff 1992; Stearns 1992). Recently though, it has been recognized that resource-based models may oversimplify the proximate basis of life-history constraints (Barnes and Partridge 2003; Isaksson et al. 2011) and poorly explain the individual variation observed in life-history strategies both within and between sexes (Isaksson et al. 2011). Therefore, a more complete understanding of the evolution of life-history trade-offs requires detailed knowledge of their mechanistic basis (Lessells 2008). Recently, it has been argued that the direct (i.e., oxidative damage) and indirect (i.e., resources required for protection) costs of oxidative stress may represent a

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general mechanism that is capable of mediating a range of important life-history trade-offs in plants and animals (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011).

Oxidative stress occurs when the production of reactive oxygen species (ROS), which are produced as by-products of oxidation–reduction (or REDOX) reactions, is elevated relative to antioxidant defenses. Although ROS are vital to normal physiological functioning (D'Autreaux and Toledano 2007; Dickinson and Chang 2011), when elevated they can oxidize and damage cellular components (e.g., DNA, RNA, proteins, and lipids) and can induce detrimental effects on organismal performance (Finkel and Holbrook 2000; Lin and Beal 2006). Consequently, managing oxidative stress represents an intricate balance between meeting the functional demands for ROS (e.g., as signaling molecules) and preventing and/or repairing any oxidative damage that results when ROS levels exceed circulating antioxidant defenses (Dowling and Simmons 2009; Monaghan et al. 2009). High levels of ROS will not necessarily result in oxidative stress if this can be balanced by the upregulation of antioxidant defenses (Monaghan et al. 2009). Likewise, relatively high levels of antioxidants will not automatically place an individual in a better REDOX state, as this will depend on the level of ROS that these defenses are required to neutralize (Monaghan et al. 2009). An understanding of oxidative stress therefore requires that both sides of this equilibrium are taken into consideration, although this approach is rarely taken in most empirical studies (Monaghan et al. 2009). This is particularly true because this balance is likely to be highly dynamic across an organism's lifetime, varying with developmental stage (Blount et al. 2003) and levels of activity (Costantini et al. 2006), as well as with exposure to stressors (Mittler 2002), pathogens (Costantini and Möller 2009), and the quantity of antioxidants in the diet (Cohen et al. 2009). As ROS are produced as an inevitable consequence of aerobic metabolism, managing oxidative stress is likely to be a major determinant of an organism's life history (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011) that should, in theory, be traded against other important life-history traits (McNamara and Buchanan 2005; Yearsley et al. 2005). Yet, despite recent calls to do so (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011), few studies have directly tested the role that oxidative stress plays in mediating important life-history trade-offs.

Ageing is an important component of the life history of most organisms and it has been proposed that oxidative stress may play a central role in the evolution of life span and ageing (Harman 1956). Ageing is the progressive, endogenous decline in the physiological and homeostatic functioning of an organism (Finch 1990) that leads to an increased risk of death with age (Rose 1991; Charlesworth 1994). Evolutionary theories of ageing hinge on the fact that the strength of natural selection declines with age (Medawar 1946, 1952; Williams 1957). This promotes the accumulation of alleles with negative effects on fitness late in life (mutation accumulation, Medawar 1952) and those that enhance early-life fitness at the expense of fitness later in life (antagonistic pleiotropy, Williams 1957). Although there is a well-developed body of evolutionary theory explaining why we age (reviewed by Hughes and Reynolds 2005), our understanding of the mechanistic basis of ageing is much less complete (Partridge and Gems 2006). The Free Radical Theory of Ageing (FRTA) (Harman 1956) was the first model to make a direct conceptual link between the production of ROS and the process of ageing. This theory posits that it is the accumulation of oxidative damage, under prolonged or chronic oxidative stress, that causes the functional decline characteristic of ageing (Harman 1956) and that differences in life span may be explained by different rates of ROS production, antioxidant defense, or repair capabilities (Harman 1992). Although there is substantial correlative evidence to support this theory (reviewed by Beckman and Ames 1998; Sohal et al. 2002), experimental manipulation of antioxidant potential has repeatedly failed to show a clear effect on life span, leading many to question the general role of oxidative stress in mediating the ageing process (Jang and Remmen 2009; Pérez et al. 2009; Speakman and Selman 2011).

The relationship between oxidative stress and life span is likely to be complex (Speakman and Selman 2011) and life-history theory predicts that variation in investment in reproductive effort is likely to be an important determinant of how animals manage oxidative stress (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011). The trade-off between reproductive effort early in life and life span is central to evolutionary theories of ageing (Williams 1957) and differences in the sign and/or strength of this relationship are likely to change how individuals manage their oxidative stress (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Isaksson et al. 2011). If antioxidants or their precursors are allocated to reproductive effort, as has been shown for carotenoid-based sexual displays (Blount et al. 2003; Pike et al. 2007) and offspring production and parental care (Wiersma et al. 2004; Losdat et al. 2011; Christe et al. 2012), a trade-off may occur between investment in antioxidant defenses and reproductive effort. This may result in a decreased life span or more rapid ageing if antioxidants are allocated to reproductive effort at the expense of somatic maintenance (Monaghan et al. 2009). Indeed, theory suggests that this may be favored if an increase in reproductive effort improves reproductive success more than a longer life does (Kokko 1997). Oxidative stress could also mediate a trade-off between reproductive effort and life span if an increase in reproductive effort elevates metabolic rate (Kavanagh 1987; Ernsting and Isaaks 1991). As ROS are produced as a by-product of aerobic

respiration, increased metabolic rate could increase the production of ROS by the mitochondria. This elevated generation of ROS could push cells into oxidative stress, leading ultimately to accelerated ageing and/or a reduction in life span. However, although metabolic rate negatively correlates with life span in a number of species (e.g., Okada et al. 2011; Seyahooei et al. 2011), greater metabolic rate does not consistently increase the rate of ROS production (Barja 2007; Hulbert et al. 2007; Galtier et al. 2009).

Sexual selection is also expected to alter the trade-off between reproductive effort and life span differentially in the sexes because females typically invest more heavily in reproduction than do males (Trivers 1972). Females are expected to pursue a reproductive strategy with moderate rates of return over a prolonged time period because their reproductive success relies on having time to acquire resources and provision offspring (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). In contrast, male reproductive success depends on the number of matings that can be achieved, and so males are expected to trade current mating success against a long life (Promislow 2003; Graves 2007; Bonduriansky et al. 2008). This pattern means that males should, on average, age faster and live shorter lives than females (Promislow 2003; Graves 2007; Bonduriansky et al. 2008) and this has been shown in a range of taxa (Comfort 1979; Finch 1990; Promislow and Harvey 1990). However, if males increase their reproductive effort with age (e.g., Mysterud et al. 2004) and this leads to an increase in reproductive success, sexual selection will promote the evolution of slower ageing and longer life spans in males. Therefore, if the costs of oxidative stress mediate the trade-off between reproductive effort and life span, sexual selection and its effects on age-dependent reproductive effort should be expected to generate differences in the balance between ROS production versus antioxidant defense in the sexes. To our knowledge, however, the role of sexual selection in affecting oxidative stress across the sexes has yet to be tested empirically in both males and females, although a number of studies have shown that the sexes do not respond equally to a perturbation in either ROS or antioxidant resources (e.g., Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Magwere et al. 2006).

In this regard, field crickets have proven to be an important model for ageing research as both male and female reproductive effort can be easily quantified. Female reproductive effort can be measured as the number of eggs produced (Head et al. 2005; Hunt et al. 2006; Zajitschek et al. 2007) and male reproductive effort can be measured as the time spent calling to attract a mate (Hunt et al. 2004; 2006; Zajitschek et al. 2007; Judge et al. 2008). In a number of cricket species, the number of females attracted to a calling male on a given night is positively correlated with his calling effort (Bentsen et al. 2006; Jacot et al. 2008; Rodríguez-Muñoz et al. 2010) and is therefore an important determinant of male reproductive success. Furthermore, as calling is metabolically costly (Kavanagh 1987), nightly calling effort also represents an important form of male reproductive effort. Recently, we used inbred lines of the decorated cricket, *Gryllodes sigillatus*, to show divergent schedules of age-dependent reproductive effort, as well as life span and ageing, across the sexes (Archer et al. *in press*). In agreement with sexual selection theory, we found that reproductive effort increased with age in males but decreased with age in females, and that males lived longer and aged more slowly than females. These divergent life-history strategies were mediated by a trade-off between early-life reproductive effort (ERE) and ageing rate in both sexes providing support for the antagonistic pleiotropy theory of ageing, although this relationship was much stronger in females than males.

Here, we examine the role of oxidative damage and antioxidant protection in shaping the evolution of sex differences in life span and ageing in *G. sigillatus*. Using the same inbred lines, we relate measures of oxidative damage (protein carbonylation) and antioxidant protection (total antioxidant capacity [TAC]) taken early and late in adulthood to life span and ageing parameters, as well as patterns of age-dependent reproductive effort, in the sexes. If the management of oxidative stress is important to the evolution of life span ageing in this species, we predict that oxidative damage and antioxidant protection will have a genetic basis and differ across the sexes, with oxidative damage being greater in the shorter lived sex (females). We also predict that oxidative damage will accumulate over time (Harman 1956) and that greater reproductive effort early in life will be associated with greater oxidative damage, at least in females. Each of these predictions comes with the obvious proviso that the predicted increase in oxidative damage is not associated with the upregulation of antioxidant defenses in this species. We discuss our findings in relationship to the role that the management of oxidative stress plays in the evolution of sex-specific differences in life span and ageing in *G. sigillatus*.

Materials and Methods

CRICKET MAINTENANCE AND THE DERIVATION OF INBRED LINES

Gryllodes sigillatus used in this study were descended from approximately 500 adult crickets collected from New Mexico in 2001. These founding animals were allowed to breed and are referred to as the outbred "USA" population. Nine inbred lines were created from this original stock. To create these lines, designated A–I, crickets selected at random from this outbred population were subjected to over 23 generations of full-sib mating and then 12 generations of panmixis within each line. Eight of these inbred lines (A, B, D–I) were used in this study and maintained as in Archer et al. (*in press)*.

EXPERIMENTAL DESIGN

Previously, we measured the genetic variation in and covariation between measures of age-dependent reproductive effort, life span, and ageing within and between the sexes using our inbred lines of *G. sigillatus* and full details of the methods are provided in Archer et al. (*in press*) (see Supporting Information for a brief summary of these methods).

In our current study, we established an additional 100 nymphs from each of the inbred lines, as well as the outbred population, in individual containers on the day they hatched and reared them to adulthood following the protocol outlined above. These crickets were taken from the same generation as those used in Archer et al. (*in press*) described above. At eclosion to adulthood, crickets were randomly assigned to one of two treatments, which dictated the age (12 or 32 days posteclosion) at which oxidative damage and protection from such damage was measured. On day 8 post eclosion, all crickets were paired at random with an outbred cricket of the opposite sex taken from the stock population for 48 h to allow mating. For crickets assayed at day 32 post eclosion, this process was repeated every 10 days with another randomly chosen stock animal of the opposite sex being provided. This was done to ensure that females did not become sperm limited during our experiment, as well as to reflect the fact that older individuals are likely to mate more than younger individuals. Immediately after mating, females were provided with moist sand in a petri dish to lay eggs. Each egg pad was left with the females for 48 h and then replaced with a new one. After their first mating period, females always had access to an egg pad unless they were with a mate and males were free to produce advertisement calls and spermatophores (which are frequently produced and discarded if mating does not occur). Thus, reproductive effort was produced continuously by males and females in our experiment, not just during our defined sampling periods. On their allotted day, crickets were placed in a 1.5 mL eppendorf tube and frozen at –80°C prior to the biochemical analysis of oxidative damage and protection. The mating regime and sampling periods used in our current study were the same as those used in Archer et al. (*in press*).

In total, six crickets of each sex per age group were frozen for biochemical analysis from each of the inbred lines and the outbred population (total $N = 216$). However, after accounting for statistical outliers ($n = 3$, see below) and one batch of samples that experienced contamination ($n = 14$), we assayed a total of 199 animals. Ordinal logistic regression showed that the distribution of statistical outliers and contaminated samples did not differ with regard to line, sex, age, or the interaction between these main effects (Line (A): $\chi^2 = 0.00$, $P = 1.00$, Sex (B): $\chi^2 = 0.00$, $P = 1.00$, Age (C): $\chi^2 = 0.00$, $P = 1.00$, A × B: $\chi^2 = 4.82$, $P = 0.78$, A × C = $\chi^2 = 2.98$, $P = 0.94$, B × C: $\chi^2 = 0.00$, $P = 1.00$, A × B × C: $\chi^2 = 4.82$, $P = 0.78$).

BIOCHEMICAL ANALYSIS OF OXIDATIVE DAMAGE AND PROTECTION

All assays were completed within three months of samples being frozen at –80°C. Thawed whole crickets were homogenized in 1 mL of phosphate buffer (pH 7.4) and then centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant fraction was separated from fat and cuticle and centrifuged for a further 5 min. This final supernatant fraction was used for all subsequent biochemical analyses. We measured the degree of oxidative damage in our samples by measuring the concentration of protein carbonyls (PCs) and the degree of protection against oxidative damage by measuring TAC. In each sample, we first measured protein concentration using the Bradford method (Bradford 1976) in samples diluted fivefold in homogenization buffer. Samples ran in triplicate showed that protein content was highly repeatable across repeated samples from the same cricket (repeatability ± SE: 0.95 ± 0.004). The concentration of PC was assayed in samples using a commercially available kit (Protein Carbonyl Assay Kit, Cayman Chemical, Ann Arbor, MI) after being diluted fivefold in homogenization buffer. Carbonyl groups (CO) are formed on protein side-chains when they are oxidized and are relatively stable, making them amenable to quantification and storage (Dalle-Donne et al. 2003). Moreover, because proteins serve a variety of important biological functions (e.g., as enzymes), oxidative damage to proteins is likely to be to the general detriment of cellular function (Dalle-Donne et al. 2003). The PC assay relies upon the covalent reaction between PC groups and 2,4-dinitrophenylhydrazine (DNPH), which produces the stable product 2,4-dinitrophenyl (DNP) hydrazone. DNP absorbs ultraviolet light and therefore can be quantified using a spectrophotometer (Spectramax Plus384, Molecular Devices, Sunnyvale, CA) set to 370 nm (Levine et al. 1990). The repeatability of our PC measures between samples run in duplicate was high (0.98 ± 0.002).

TAC was also measured using a commercially available kit (Antioxidant Assay Kit, Cayman Chemical), which assays the oxidation of ABTS (2,2′-Azino-di-[3-ethybenzthiazoline sulphonate]) by metmyoglobin, which is inhibited by antioxidants contained in the sample relative to Trolox controls. Oxidized ABTS product can then be measured using a spectrophotometer set to 750 nm. All samples were diluted 100-fold and run in duplicate and the repeatability of our TAC measures was high across samples (0.95 ± 0.007).

In all statistical analyses, we used the mean value for PC and TAC measures taken across our repeated measurements of a given sample. To control for differences in the body size of crickets, PC and TAC measures were expressed per milligram of protein analyzed. PC is therefore provided in units of nanomoles per milligram of protein (nmol/mg) and TAC in units of micromoles per milligram of protein (mmol/mg).

STATISTICAL ANALYSES

Prior to analysis, we $\log_{10}$ transformed our response variables (PC and TAC) to ensure normality. After transformation, we conducted a multivariate outlier analysis using Mahalanobis distances and three crickets were excluded from further analysis as they were greater than 2 SD from the mean centroid (Tabachnick and Fidell 1989). We examined the effect of line, sex, and age on PC and TAC using a mixed-model multivariate analysis of variance (MANOVA), including line as a random effect and sex and age as fixed effects in the model. All interactions between line and the fixed effects were treated as a random effect in the model (Zar 1999). We followed this MANOVA with a series of univariate general linear mixed-models (GLMMs) with the same model structure to determine how each of our response variables contributed to the overall multivariate effects. This overall model identified significant differences in PC and TAC across the sexes (see Results) and we therefore examined the effects of line and age on PC and TAC within each sex separately using a mixed-model MANOVA, including age as a fixed effect and line and line × age as random effects. As outlined above, we followed these MANOVAs with a series of univariate GLMMs to determine how each of our response variables contributed to the overall multivariate effects. All models were implemented in SAS (version 9.1.3, SAS Institute Inc., Cary, NC, USA) using the "PROC GLM" procedure with the appropriate F values and degrees of freedom being specified according to Zar (1999, Appendix A). In no instance did model simplification alter the outcome of the model, so we present the complete model for each analysis. All data are presented as mean ± 1 SE.

The use of inbred organisms in evolutionary research can be problematic, particularly if inbreeding depression results in a large reduction in the fitness of individuals and/or if the inbred lines do not represent a true random sample of genotypes from the outbred population from which they were derived (Hoffmann and Parsons 1988). Therefore, in addition to the inbred lines, we also assayed PC and TAC in a sample of crickets from the outbred population they were derived from. In doing this, our aim was not to test how inbreeding influences PC and TAC (which would require replicate outbred populations), rather to provide a baseline for comparison. For this reason, these outbred crickets are not included in any of our statistical analyses but are provided in our figures for visual comparison.

Quantitative genetic analyses

We estimated the heritabilities of PC, TAC, and life-history traits (reproductive effort, life span, and ageing) from our inbred lines by calculating the intraclass correlation coefficient (t) from the within and between line variance components extracted from a general linear model that included line as a random effect (Hoffmann and Parsons 1988; David et al. 2005). The SE of this estimate (SE(t)) was calculated according to Becker (1984). These estimates of t and SE(t) were then used to calculate the heritability and SE for each trait following the protocol outlined in Hoffmann and Parsons (1988). We estimated genetic correlations (and their SEs) between traits using the "delete-one" jackknife method of Roff and Preziosi (1994). This approach provides a more accurate estimate of this genetic parameter than those based on conventional inbred line means when fewer than 20 inbred lines are used in the analysis (Roff and Preziosi 1994). A full description on using these approaches to estimate genetic parameters in our inbred lines is provided in Archer et al. (*in press*). We considered our estimates of heritability and genetic correlations statistically significant if the estimates divided by their SEs were greater than 1.96, allowing us to reject the null hypothesis of no correlation based on a two-tailed t-distribution with infinite degrees of freedom. These estimates of genetic (co)variance from inbred lines contain variance due to dominance and/or epistasis and therefore should be considered broad-sense estimates (Falconer and Mackay 1996). As crickets were reared individually, the variance between lines due to common environment effects was minimized in our experimental design.

Results

A mixed-model MANOVA indicated that line and sex significantly influenced the linear combination of PC and TAC measures (Table 1). There was, however, no overall multivariate effect of age on levels of PC and TAC (Table 1). Univariate GLMMs showed that the overall multivariate effect of line was driven by significant line differences in both PC and TAC (Table 1). Likewise, the overall multivariate effect of sex was due to sex differences in both PC and TAC (Table 1) with females, on average, exhibiting higher levels of both PC and TAC than males (Fig. 1). The magnitude of sex differences in PC and TAC, however, varied across lines and with age, as indicated by the significant line × sex and age × sex interactions (Table 1). Consequently, to facilitate subsequent interpretation we conducted separate analyses on males and females.

In males, there was a significant multivariate effect of line and age on the linear combination of PC and TAC but no interaction between these main effects (Table 2A). GLMM showed that the overall multivariate effect of line resulted from significant line differences in both PC and TAC (Fig. 2A and B; Table 2A). In contrast, the overall multivariate effect of age was due to the fact that TAC significantly increased with age in males whereas PC did not (Fig. 2A and B; Table 2A). In females, there was a significant multivariate effect of line on the linear combination of PC and TAC but there was no effect of age nor was there an interaction between line and age (Fig. 2C and D; Table 2B). As shown for males, GLMM revealed that the overall multivariate effect of line

Table 1. Mixed-model MANOVA examining the effects of line, sex, age, and their interactions on levels of protein carbonylation (PC) and total antioxidant capacity (TAC) per mg protein in *Gryllodes sigillatus*. We also present univariate general linear mixed-models to determine how each of these response variables contributed to the overall multivariate effect. As there were significant differences in levels of PC and TAC across the sexes, as illustrated by a significant main effect of sex as well as interactions with line and age, we conducted subsequent analyses for each sex.

	MANOVA			
	Wilks' λ	*F*-value	df	*P*-value
Line (A)	0.675	4.379	14,282	0.0001
Sex (B)	0.026	111.446	2,6	0.0001
Age (C)	0.781	0.845	2,6	0.476
A × B	0.850	1.812	14,282	0.037
A × C	0.907	1.001	14,282	0.451
B × C	0.413	5.229	2,6	0.048
A × B × C	0.911	0.964	14,282	0.490
	Univariate GLMM			
	F-value	df	*P*-value	
PC				
A	4.483	7,7.869	0.0265	
B	42.809	1,7.015	0.0003	
C	1.291	1,7.038	0.294	
A × B	4.428	7,7	0.034	
A × C	1.725	7,7	0.246	
B × C	1.090	1,7.066	0.331	
A × B × C	0.722	7,142	0.657	
TAC				
A	10.010	7,7.034	0.004	
B	202.867	1,7.059	0.0001	
C	1.212	1,7.069	0.307	
A × B	0.709	7,7	0.675	
A × C	0.601	7,7	0.742	
B × C	9.122	1,7.041	0.019	
A × B × C	1.144	7,142	0.340	

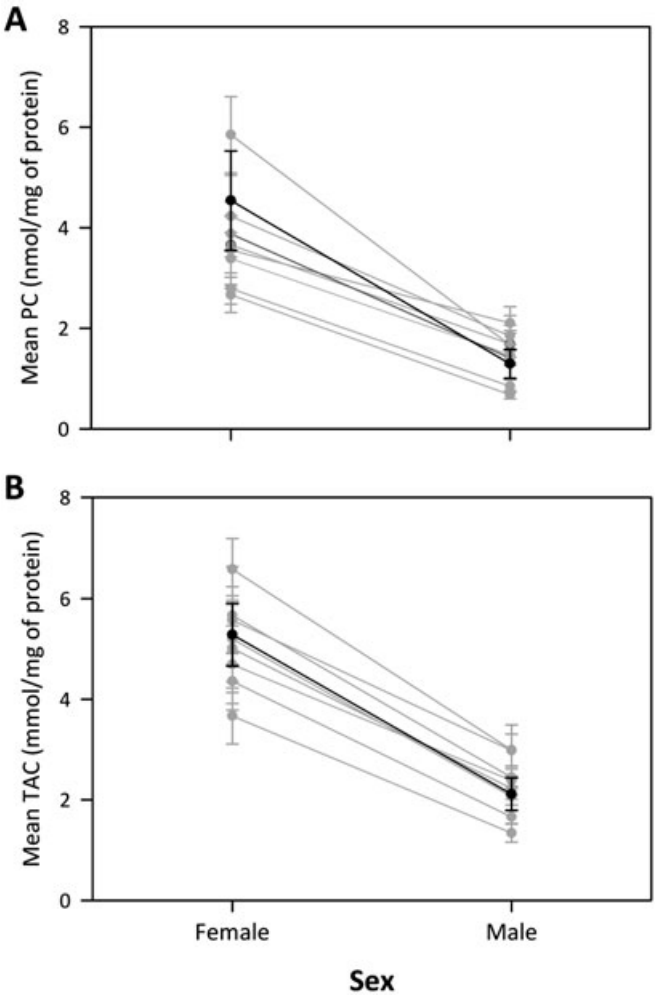

Figure 1. Mean (±SE) sex differences in protein carbonylation (PC) and total antioxidant capacity (TAC) in the different inbred lines and the outbred population of *Gryllodes sigillatus*. The outbred population is provided in black and the inbred lines in light gray.

detected in females was due to significant line differences in both PC and TAC (Fig. 2C and D; Table 2B).

Consistent with these line differences, PC and TAC measured early and late in life were significantly heritable in both sexes and these measures were positively genetically correlated within each sex, with the exception of early and late TAC in females (Table 3). In our previous study (Archer et al. *in press*), we found that measures of age-dependent reproductive effort, life span, and ageing all exhibited significant heritabilities in the sexes (Table 3). Moreover, we found a significant positive genetic correlation between ERE and the rate of ageing (β) in both sexes, albeit this relationship was considerably stronger in females (Table 3). Here, we show that both PC and TAC are genetically correlated with reproductive effort, although the sign and strength of these genetic correlations varied with age and across the sexes (Table 3). In males, there were consistent negative genetic correlations between ERE and late-life reproductive effort (LRE) and both PC and TAC, with the exception of PC and reproductive effort expressed late in life (Table 3). In females, there was a negative genetic correlation between ERE and both PC and TAC when expressed early in life but a positive genetic correlation when expressed later in life (Table 3). There were, however, consistent negative genetic correlations between LRE and both PC and TAC, irrespective of when they were expressed in life (Table 3).

We also found that PC and TAC were genetically correlated with life span and measures of ageing in the sexes. Irrespective

Table 2. **Mixed-model MANOVA examining the effects of line and age on levels of PC and TAC in (A) male and (B) female *Gryllodes sigillatus*. We also present univariate general linear mixed-models to determine how each response variable contributes to the overall multivariate effect.**

	MANOVA			
A. Males	Wilks' λ	*F*-value	df	*P*-value
Line (A)	0.467	4.562	14,138	0.0001
Age (B)	0.395	5.178	2,6	0.049
A × B	0.815	1.061	14,138	0.398
	Univariate GLMM			
	F-value	df	*P*-value	
PC				
A	5.192	7,7	0.023	
B	1.893	1,7.085	0.211	
A × B	1.289	7,70	0.274	
TAC				
A	6.893	7,7	0.010	
B	10.634	1,7.111	0.013	
A × B	0.989	7,70	0.454	
	MANOVA			
B. Females	Wilks' λ	*F*-value	df	*P*-value
Line (A)	0.729	2.199	14,142	0.010
Age (B)	0.720	1.177	2,6	0.373
A × B	0.846	0.892	14,142	0.574
	Univariate GLMM			
	F-value	df	*P*-value	
PC				
A	4.182	7,7	0.039	
B	0.100	1,7.045	0.766	
A × B	0.708	7,72	0.672	
TAC				
A	3.873	7,7	0.047	
B	2.651	1,7.036	0.147	
A × B	0.875	7,72	0.536	

of whether expressed early or late in life, there were consistent negative genetic correlations between life span and levels of PC and TAC in both males and females (Table 3). In females, levels of PC and TAC expressed early in life were positively genetically correlated with baseline mortality, whereas later in life these measures were positively genetically correlated with the rate of ageing (Table 3). In males, there were consistent positive genetic correlations between baseline mortality and the levels of both PC and TAC expressed early and late in life (Table 3). However, there was only a positive genetic correlation between the level of TAC expressed late in life and the rate of ageing in males (Table 3).

Previously, we have shown strong positive intersexual genetic correlations between measures of age-dependent reproductive effort, life span, and measures of ageing that suggests these traits are unlikely to evolve independently in the sexes (Table 4; Archer et al. *in press*). Consistent with this, we found that the genetic correlations between measures of age-dependent PC and TAC were generally strong and positive across the sexes, with the exception of PC measured late in life (Table 4). The patterns of genetic correlations between these traits across the sexes largely mirrored the patterns observed within each sex (Table 4). Notable exceptions include the strong positive genetic correlation between baseline mortality and PC expressed late in life across the sexes, as well as the positive genetic correlation between PC expressed early in life in females and LRE in males (Table 4).

Discussion

Despite tremendous progress in biogerontology (Partridge 2010), we still have a relatively poor understanding of the proximate basis of ageing (Partridge and Gems 2006). This is especially true for the role that oxidative stress plays in mediating the ageing process (D'Autreaux and Toledano 2007). In the decorated cricket, *G. sigillatus*, the sexes have contrasting patterns of age-dependent reproductive effort with male calling effort increasing with age, whereas female fecundity decreases with age (Archer et al. *in press*). As predicted by sexual selection theory (Promislow 2003; Graves 2007; Bonduriansky et al. 2008), these contrasting schedules of reproductive effort are associated with differences in life span and ageing between the sexes, with males living longer and ageing more slowly than females (Archer et al. *in press*). These divergent life-history strategies are mediated by a positive genetic correlation between ERE and ageing rate in both sexes, although this relationship is considerably stronger in females (Archer et al. *in press*). Here, we examined whether oxidative stress is a proximate cost of reproductive effort that is able to mediate these sex-specific rates of ageing and differences in life span in male and female *G. sigillatus*. We show that levels of oxidative damage to proteins (protein carbonylation) and antioxidant protection (TAC) are greater in females than in males and that both measures possess significant levels of genetic variation in the sexes. Moreover, these measures were shown to be genetically correlated with life span, measures of ageing, and age-dependent reproductive effort. These relationships were complex, however, with the sign and magnitude differing with age and across the sexes. Thus, while our findings suggest that the management of oxidative stress is likely to play an important role in the evolution of life span and ageing in *G. sigillatus*, the exact nature of this role is currently difficult to determine precisely.

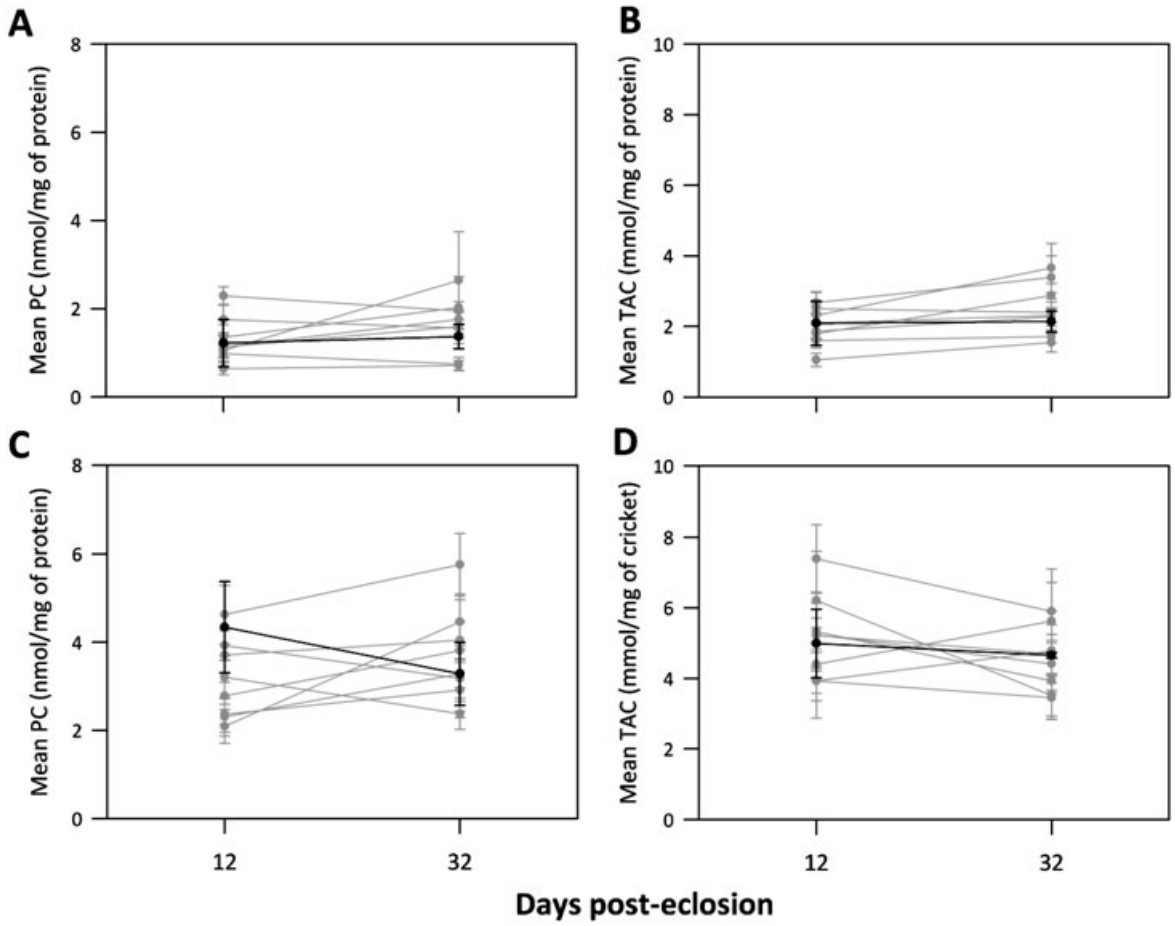

Figure 2. Age-dependent protein carbonylation (PC) and total antioxidant capacity (TAC) in the different inbred lines (light gray) and the outbred population (black) of (A and B) male and (C and D) female *Gryllodes sigillatus*.

The FTRA (Harman 1956) is undoubtedly the best supported mechanistic theory of ageing (Beckman and Ames 1998; Finkel and Holbrook 2000). It predicts that the accumulation of oxidative damage to proteins, lipids, and DNA, which occurs when the production of ROS exceeds antioxidant defense and repair, causes ageing (Harman 1956). Thus short-lived animals should have high levels of oxidative damage due to a chronic imbalance between ROS production and endogenous protection and repair systems (Beckman and Ames 1998). Over the last 50 years, this theory has gained a great deal of empirical support (reviewed in Beckman and Ames 1998; Sohal et al. 2002; Robert et al. 2007; Speakman and Selman 2011). Recently, however, the support for this theory has somewhat waned, specifically in light of the findings that deletion (or overexpression) of specific antioxidant enzymes (either singly or in combination) has been shown to have an inconsistent effect on life span in a number of laboratory model species (Doonan et al. 2008; Jang and Remmen 2009; Pérez et al. 2009; Speakman and Selman 2011). In addition, it is clear that using antioxidant supplementation protocols or genetic manipulations that increase antioxidant capacity has equivocal effects on oxidative damage, metabolic health, and life span (e.g., Ristow et al. 2009; Cabreiro et al. 2011). In fact, in many cases antioxidant expression may be directly linked to ROS production. For example, in *Arabidopsis* (Desikan et al. 2001) and *Caenorhabditis elegans* (Schulz et al. 2007), ROS influences the expression of a number of genes that encode important antioxidant enzymes. This regulation of antioxidant enzyme expression by ROS is one mechanism that allows cells to precisely regulate their REDOX state and explains why antioxidants are often positively correlated with ROS production and/or oxidative damage (Costantini and Verhulst 2009) and negatively correlated with life span (Buttemer et al. 2010). Furthermore, when optimal REDOX states are perturbed, as occurs during antioxidant manipulation, cellular function may also be impaired (Lin and Beal 2006; Tapia 2006) resulting in pathologies that can reduce life span (Magwere et al. 2006). For example, in humans, antioxidant supplementation can ameliorate the positive health effects of exercise because ROS are vital signaling molecules that induce the beneficial phenotype (Ristow et al. 2009). Consequently, using the failure of antioxidant

Table 3. Heritabilities (along diagonal) and genetic correlations (below diagonal) for early and late-life reproductive effort (ERE and LRE), life span, Gompertz ageing parameters (α and β), early and late-life protein carbonylation (EPC and LPC), and early and late-life total antioxidant capacity (ETAC and LTAC) in male and female *Gryllodes sigillatus*. SEs for these estimates are provided in parentheses. Values given in bold are significant at $P < 0.05$ and those highlighted in gray are taken directly from Archer et al. (*in press*).

	ERE	LRE	LS	α	β	EPC	LPC	ETAC	LTAC
Males									
ERE	**0.36**								
	(0.14)								
LRE	**0.15**	**0.52**							
	(0.07)	**(0.14)**							
LS	0.35	**0.49**	**0.97**						
	(0.24)	**(0.05)**	**(0.02)**						
α	**−0.44**	**−0.40**	**−0.79**	**0.99**					
	(0.10)	**(0.05)**	**(0.03)**	**(0.01)**					
β	**0.44**	−0.06	**−0.42**	−0.26	**0.99**				
	(0.17)	(0.14)	**(0.10)**	(0.40)	**(0.01)**				
EPC	**−0.74**	**−0.34**	**−0.55**	**0.87**	−0.68	**0.77**			
	(0.05)	**(0.03)**	**(0.07)**	**(0.00)**	(0.61)	**(0.11)**			
LPC	**−0.48**	0.30	−0.19	0.12	−0.04	**0.39**	**0.43**		
	(0.14)	(0.46)	(0.22)	(0.13)	(0.06)	**(0.10)**	**(0.18)**		
ETAC	**−0.72**	**−0.23**	**−0.87**	**0.80**	0.05	**0.80**	**0.58**	**0.54**	
	(0.19)	**(0.02)**	**(0.01)**	**(0.01)**	(0.25)	**(0.01)**	**(0.11)**	**(0.17)**	
LTAC	**−0.46**	**−0.28**	**−0.69**	**0.39**	**0.46**	**0.38**	**0.53**	**0.78**	**0.76**
	(0.12)	**(0.03)**	**(0.04)**	**(0.13)**	**(0.06)**	**(0.13)**	**(0.10)**	**(0.02)**	**(0.11)**
Females									
ERE	**0.48**								
	(0.14)								
LRE	0.34	**0.46**							
	(0.21)	**(0.14)**							
LS	−0.11	0.09	**0.94**						
	(0.11)	(0.11)	**(0.03)**						
α	**−0.98**	−0.02	**−0.40**	**0.98**					
	(0.45)	(0.04)	**(0.03)**	**(0.01)**					
β	**0.97**	−0.00	0.09	**−1.16**	**0.99**				
	(0.06)	(0.16)	(0.15)	**(0.23)**	**(0.00)**				
EPC	**−0.25**	**−0.46**	**−0.37**	**0.24**	−0.11	**0.38**			
	(0.03)	**(0.21)**	**(0.18)**	**(0.06)**	(0.10)	**(0.18)**			
LPC	**0.16**	**−0.60**	**−0.61**	−0.05	**0.26**	**0.91**	**0.52**		
	(0.08)	**(0.06)**	**(0.02)**	(0.22)	**(0.02)**	**(0.19)**	**(0.17)**		
ETAC	−0.01	**−0.42**	−0.30	**0.13**	0.02	**0.90**	**0.92**	**0.33**	
	(0.01)	**(0.13)**	(0.25)	**(0.06)**	(0.03)	**(0.07)**	**(0.13)**	**(0.15)**	
LTAC	**0.12**	−0.08	**−0.80**	0.15	**0.07**	**0.72**	**0.71**	0.46	**0.34**
	(0.02)	(0.17)	**(0.02)**	(0.16)	**(0.02)**	**(0.11)**	**(0.12)**	(0.34)	**(0.17)**

manipulations as evidence against the FRTA should always be interpreted with a degree of caution (Lin and Beal 2006).

In fact, the results we present here provide two lines of support for the FRTA. First, we found that oxidative damage was significantly greater in females; the shorter lived and most rapid ageing sex in *G. sigillatus*. Sex differences in life span are widespread (Promislow 1992; Clutton-Brock and Isvaran 2007) and several studies have shown that oxidative damage and/or ROS production is greater in the shorter lived sex (e.g., Ide et al. 2002; Borrás et al. 2003; Ali et al. 2006). Oxidative stress may differ across the sexes because of differences in metabolic rate or mitochondrial efficiency, which may affect rates of ROS production (Magwere et al. 2006). Alternatively, the sexes may show different responses to changes in REDOX status that affect their resistance to oxidative damage (Magwere et al. 2006). Both of these hypotheses have gained some empirical support in the

Table 4. Intersexual genetic correlations (±SE) for life span, Gompertz ageing parameters, and age-dependent reproductive effort in *Gryllodes sigillatus*. Abbreviations are as outlined in Table 3.

♂ \ ♀	ERE	LRE	LS	α	β	EPC	LPC	ETAC	LTAC
ERE	0.35	**0.24**	**0.31**	**−0.61**	0.51	−0.29	−0.09	**−0.36**	−0.18
	(0.21)	**(0.07)**	**(0.09)**	**(0.21)**	(0.38)	(0.18)	(0.07)	**(0.09)**	(0.24)
LRE	−0.07	**0.15**	**0.85**	**−0.39**	0.13	**0.17**	**−0.24**	0.19	**−0.41**
	(0.16)	**(0.07)**	**(0.03)**	**(0.03)**	(0.27)	**(0.04)**	**(0.08)**	(0.11)	**(0.12)**
LS	**−0.10**	0.08	**0.88**	**−0.33**	**0.08**	**−0.76**	**−0.79**	**−0.63**	**−0.94**
	(0.05)	(0.17)	**(0.00)**	**(0.09)**	**(0.04)**	**(0.10)**	**(0.05)**	**(0.20)**	**(0.01)**
α	**−0.57**	**−0.37**	**−0.71**	**0.76**	**−0.66**	**0.79**	**0.71**	**0.65**	**0.69**
	(0.14)	**(0.10)**	**(0.01)**	**(0.02)**	**(0.24)**	**(0.07)**	**(0.19)**	**(0.16)**	**(0.06)**
β	**0.92**	**0.30**	**−0.28**	−0.95	**0.94**	0.18	**0.44**	**0.19**	**0.51**
	(0.38)	**(0.13)**	**(0.14)**	(0.93)	**(0.23)**	(0.12)	**(0.03)**	**(0.09)**	**(0.05)**
EPC	**−0.78**	**−0.09**	**−0.53**	**0.99**	**−1.04**	**0.48**	0.25	**0.44**	**0.34**
	(0.38)	**(0.04)**	**(0.03)**	**(0.03)**	**(0.44)**	**(0.06)**	(0.21)	**(0.09)**	**(0.12)**
LPC	0.06	**0.23**	0.10	**0.27**	**−0.27**	**0.31**	0.15	**0.54**	−0.11
	(0.09)	**(0.03)**	(0.46)	**(0.08)**	**(0.02)**	**(0.10)**	(0.11)	**(0.10)**	(0.21)
ETAC	−0.15	0.01	**−0.69**	**0.58**	**−0.41**	**0.76**	**0.62**	**0.74**	**0.71**
	(0.17)	(0.14)	**(0.02)**	**(0.01)**	**(0.13)**	**(0.05)**	**(0.09)**	**(0.03)**	**(0.04)**
LTAC	**0.47**	0.21	**−0.63**	0.04	**0.20**	0.40	**0.58**	**0.68**	**0.48**
	(0.08)	(0.42)	**(0.03)**	(0.10)	**(0.06)**	(0.25)	**(0.10)**	**(0.05)**	**(0.08)**

literature. For example, in certain strains of rats, females live longer than males, release fewer ROS from their mitochondria, and have a higher expression of antioxidant enzymes due to upregulation by the sex hormone oestrogen (Borrás et al. 2003; 2007; Viña et al. 2003). The mechanistic basis of sex differences in oxidative stress has been less thoroughly explored in invertebrates. The greater oxidative damage we show in female crickets may be because sexual selection, which is likely to promote shorter lives and faster ageing in females than males (Archer et al. *in press*), has driven the evolution of sex differences in levels of mitochondrial efficiency, the activity of key antioxidant enzymes, and/or rates of repair of oxidised molecules. Such differences have recently been shown across short- and long-lived ecotypes of the garter snake (*Thamnophis elegans*) that have evolved under different rates of extrinsic mortality (Robert and Bronikowski 2010). However, to our knowledge, there is currently no direct empirical evidence to suggest that sexual selection can generate similar differences across the sexes.

The second line of support that we found for the FRTA was a negative genetic correlation between life span and oxidative damage in both sexes, although the magnitude of this genetic correlation differed across the sexes and with age. In females, oxidative damage assayed early and late in life was negatively correlated with life span but in males, only damage measured early in life was negatively genetically correlated with life span. Although a number of studies have shown negative phenotypic correlations between life span and oxidative damage and/or rate of ROS production (Sohal et al. 1993a,b, 1995; Lambert et al. 2007; Robert et al. 2007), few have explored the genetic basis of this relationship. In *Drosophila*, divergent artificial selection on life span results in correlated changes in the expression and activity of particular antioxidant enzymes, as well as survival resistance to oxidative stress (Arking et al. 2000; Harshman and Haberer 2000). Furthermore, QTL mapping studies using recombinant inbred lines of *Drosophila* have shown that QTLs for survival resistance to oxidative stress are relatively abundant and colocalize the same region of the genome as those for life span (Curtsinger and Khazaeli 2002; Wang et al. 2006). Collectively, these studies demonstrate that resistance to oxidative stress is heritable and genetically correlated with life span in *Drosophila*, a finding that we now provide empirical support for in *G. sigillatus.*

In addition to this negative genetic correlation between life span and oxidative damage, we also found that oxidative damage was genetically correlated with ageing parameters. In both sexes, we found positive genetic correlations between baseline mortality and oxidative damage early in life. This may arise because lines with higher baseline mortality, which is generally interpreted as a measure of the overall "frailty" of a population (Pletcher et al. 2000), generate more free radicals during metabolism (Speakman et al. 2004), are less robust to stressors that perturb REDOX homeostasis (Pal et al. 2010), and/or are less able to detect, repair, or remove oxidized molecules (Caple et al. 2010;

Tyson et al. 2010). In addition, we show that oxidative damage late in life was positively genetically correlated with the rate of ageing in females, but not in males. We have previously shown that males increase their investment in reproductive effort with age whereas females show reproductive senescence (Archer et al. *in press*). As a result of these patterns of reproductive investment, males may be under stronger selection to combat ageing and, as a result, oxidative stress. Selection for a long life and reduced oxidative stress will be less pronounced in females, who show low rates of reproductive effort later in life (Archer et al. *in press*). Although our experiment does not allow us to determine whether it is oxidative damage that accelerates ageing or the ageing process that generates higher levels of oxidative damage, it does provide a potential proximate mechanism explaining the faster rate of ageing in females that we have already documented in this species (Archer et al. *in press*).

We also found sex-specific differences in the relationships between age-dependent reproductive effort, oxidative damage, and antioxidant protection. In males, there were consistent negative genetic correlations between age-dependent reproductive effort and measures of oxidative damage and antioxidant protection. The negative genetic correlation between reproductive effort and oxidative damage may reflect the fact that only high-quality males (superior genotypes) are able to invest heavily in reproductive effort. These males may be able to call more to attract females without incurring elevated oxidative damage because they produce fewer ROS per unit oxygen consumed (i.e., have more efficient mitochondria) or because they are better able to repair any oxidative damage. Such differences in mitochondrial efficiency (Borrás et al. 2003, 2007; Viña et al. 2003; Magwere et al. 2006) and rates of repair of oxidative damage (Robert and Bronikowski 2010) have been shown to account for variation in oxidative stress and life span within a number of species. We also observed similar negative genetic correlations between these traits in females, with the notable exception that there was a positive genetic correlation between ERE and both measures of oxidative damage and antioxidant protection late in life. Moreover, there were positive genetic correlations between measures of oxidative damage and antioxidant protection late in life and the rate of ageing in females, suggesting that the management of oxidative stress may play an important role in mediating the much stronger antagonistic pleiotropy between ERE and the rate of ageing previously documented in females (Archer et al. *in press*). This may be because egg production may increase female metabolic rate relatively more than calling effort does in males. If a higher metabolic rate increases the rate of ROS production, it could push cells into oxidative stress and increase levels of oxidative damage in females. In the house cricket (*Acheta domesticus*), egg production increases female metabolic rate by a factor of 1.75 times over the basal metabolic rate (Clifford and Woodring 1986), whereas calling only increased male metabolic rate by a factor of 1.5 over basal (Hack 1998). Unfortunately, it is not known whether a higher metabolic rate generates more ROS in this species. Finally, female reproductive effort may increase oxidative damage more than male reproductive effort if females divert relatively more of their antioxidants to producing eggs than to somatic maintenance. Studies on a diverse range of taxon have shown that the intake of antioxidants increase egg production in females (e.g., insects—Jedlička et al. 2009; amphibians—Ogilvy et al. 2012; fish—Mehrad and Sudagar 2010; birds—Blount et al. 2003; Bertrand et al. 2006; mammals—Zeng et al. 2008) and that this may come at the expense of somatic maintenance (e.g., Blount et al. 2003; Morales et al. 2008), but no study, to our knowledge, has formally compared these relationships across the sexes.

Although these results offer support for the FRTA, a key prediction of this theory is that oxidative damage should accumulate over time (Harman 1956). In *G. sigillatus*, we found that oxidative damage did not accumulate with age in either males or females. Several studies have found age-associated increases in oxidative damage, including protein oxidation (e.g., Stadtman 1992; Sohal et al. 1995; Kasapoglu and Özben 2001). However, because levels of oxidative damage and/or rates of ROS production frequently vary across different tissue types (Kaneko et al. 1997; Radák et al. 2002; Borrás et al. 2003) and age-associated changes in oxidative damage may occur at different rates in different tissues (Sohal et al. 1995), there may have been age-associated changes in oxidation to other molecules (e.g., lipids or DNA) not examined in our study. Our results offer some evidence that this may be the case: although we did not find an age-dependent increase in oxidative damage, we did find that older males had greater TAC than younger males. In *G. sigillatus*, we found strong positive genetic correlations between oxidative damage and antioxidant protection. This finding has been reported in numerous other species (e.g., Costantini and Verhulst 2009; Buttemer et al. 2010) and appears to occur because animals upregulate their investment in antioxidant protection in response to greater rates of ROS production. However, upregulated antioxidant defenses may not always be sufficient to neutralize ROS production and oxidative damage may still occur (Costantini and Verhulst 2009). This appears to also be the case in *G. sigillatus*, whereby the lines and sex (females) with the greatest levels of antioxidant protection still incur the highest levels of oxidative damage, and explains the counterintuitive finding that antioxidant protection is negatively genetically correlated with life span in this species. This relationship most likely reflects the fact that shorter lived crickets produce more antioxidants to combat high levels of ROS production rather than antioxidants carrying a mortality cost per se. This positive relationship we document between oxidative damage and antioxidant protection in *G. sigillatus* also suggests that the increase in male antioxidants with age may illustrate an adaptive

response to an age-associated increase in pro-oxidant production (Harman 1972; Fleming et al. 1982). This result further highlights the importance of measuring both antioxidant protection and oxidative damage when making inferences about the management of oxidative stress (Monaghan et al. 2009).

Given the sex-specific nature of the relationships between oxidative damage, antioxidant protection, and life span that we document here, plus the divergent life-history strategies that we have demonstrated elsewhere (Archer et al. *in press*), the sexes should be under selection to manage their REDOX states very differently in *G. sigillatus*. However, we found strong positive genetic correlations across the sexes for both oxidative damage and antioxidant protection suggesting that these measures are not free to evolve independently across the sexes and raise the possibility of intralocus sexual conflict over the management of oxidative stress in this species. Intralocus sexual conflict occurs whenever selection on shared phenotypic traits in one sex displaces the other sex from its phenotypic optima and occurs when shared traits have a common genetic basis but are subject to contrasting selection (Bonduriansky and Chenoweth 2009). This, in theory, will prevent the sexes from evolving independently to their own sex-specific phenotypic optima (Bonduriansky and Chenoweth 2009). Consequently, a signature that intralocus sexual conflict is currently operating in a population, is a strong and positive intersexual genetic correlation for the shared trait(s) and fitness surfaces that differ across the sexes (Bonduriansky and Chenoweth 2009). We have previously shown the potential for intralocus sexual conflict over age-dependent reproductive effort, ageing, and life span in *G. sigillatus* (Archer et al. *in press*) and our current study shows that similar conflict may exist over the management of oxidative stress. However, direct confirmation that intralocus sexual conflict is indeed operating, as well as a direct estimate of its strength, requires further work to formally estimate the sex-specific fitness surfaces for age-dependent oxidative damage and protection in *G. sigillatus* (e.g., Lewis et al. 2011).

In conclusion, our study shows that oxidative damage and antioxidant protection are important determinants of sex-specific life span and ageing in *G. sigillatus*. In support of the FRTA, we find that oxidative damage is greater in the shorter lived sex (females), is negatively genetically correlated with life span in both sexes, and is positively genetically correlated with the rate of ageing in females. In contrast, the lack of evidence for an age-dependent increase in oxidative damage undermines a key premise of this theory. However, we found a positive association between oxidative stress and antioxidant protection, a signature of active upregulation of antioxidants in response to greater production of ROS. We also found that in females, but not males, that oxidative damage late in life is a proximate cost of early reproductive effort that accelerates rates of ageing. This indicates that oxidative stress could mediate this important, sex-specific life-history trade-off in *G. sigillatus*, as well as explaining intersexual differences in ageing and life span. Our measures of oxidative damage and antioxidant protection were positively genetically correlated across the sexes suggesting that the sexes may be constrained in their ability to manage oxidative stress. Together, our work highlights the importance of considering the important role of sexual selection and intralocus sexual conflict in research examining the mechanistic basis of ageing.

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SUMMARY OF METHODS USED IN ARCHER ET AL. (*IN PRESS*)